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MINI REVIEW

Alternative Oxidase and Uncoupling Protein: Thermogenesis Versus Cell Energy Balance

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The physiological role of an alternative oxidase and an uncoupling protein in plant and protists is discussed in terms of thermogenesis and energy metabolism balance in the cell. It is concluded that thermogenesis is restricted not only by a lower-limit size but also by a kinetically-limited stimulation of the mitochondrial respiratory chain.

KEY WORDS: Alternative oxidase; uncoupling protein, mitochondria

INTRODUCTION

Plant mitochondria present the puzzling property of possessing simultaneously two energy-dissipating systems that lead to the same final effect, i.e., a decrease in ATP synthesis yield. First, discovered in plant thermogenic tissues (Lance, 1972; Meeuse, 1975), an alternative oxidase (AOx) is branched from the main respiratory chain at the level of ubiquinone and catalyses the oxidation of ubiquinol by oxygen without formation of an electrochemical H^+ gradient (Moore and Siedow, 1991; Sluse and Jarmuszkiewicz, 1998). Therefore, AOx dissipates the redox energy into heat. Second, recently discovered in several plant tissues, a plant uncoupling protein (UCP) catalyzes a protonphoretic cycle activated by free fatty acids (Vercesi *et al.*, 1995; Ježek *et al.*, 1998). Like mammalian UCPs (UCP1, 2, 3), plant UCP dissipates directly the electrochemical H^+ gradient allowing protons to bypass the ATP synthase route thereby uncoupling respiration from phosphorylation and producing heat (Ricquier and Bouillaud, 2000).

Simultaneous presence of these two energy-dissipating systems has also been demonstrated recently in mitochondria from *Acanthamoeba castellanii* (Jarmuszkiewicz *et al.*, 1999), a non-photosynthetic soil amoeboid protozoon, which in molecular phylogenesis appears on a branch basal to the divergence points of plants,

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animals and fungi (Wainright *et al.*, 1993; Gray *et al.*, 1999). Presence of UCP has also been shown in a non-fermentative yeast *Candida parapsilosis* (Jarmuszkiewicz *et al.*, 2000). These discoveries indicate that UCP should occur in the whole eukaryotic world. On the contrary, animals have lost the AOx pathway that looks maintained in plants (Vanlerberghe and McIntosh, 1997; Wagner and Moore, 1997) and several types of microorganisms like fungi (Lambowitz *et al.*, 1989; Sakajo *et al.*, 1991), trypanosomes (Clarkson *et al.*, 1989) and amoeba (Jarmuszkiewicz *et al.*, 1999).

The only obvious physiological function of AOx and UCP can be recognized in specialized plant and animal thermogenic tissues as heat generation related to increase in temperature (thermogenesis): AOx generates heat in spadices of *Araceae* during reproductive processes (Meeuse, 1975) and UCP1 generates heat in brown adipose tissue of newborn, cold-acclimated and hibernating mammals (Nicholls and Rial, 1999). In non-thermogenic tissue or unicellulars the role of AOx and/or UCP is not yet fully understood.

The aim of this paper is to shed light on the functional connection of AOx and UCP in plant mitochondria and on their potential role in non-thermogenic tissues and unicellulars.

FREE-ENERGY ROUTES IN PLANT MITOCHONDRIA

Figure 1 shows the energy routes in plant mitochondria from succinate. The pathway: Complex II→AOx is fully redox-free energy dissipating and can be inhibited by benzohydroxamate (BHAM), an inhibitor of AOx. In the presence of BHAM, the pathway: Complex II→III→IV conserves redox energy into $\Delta\mu H^+$ that can be optimally converted into ATP (phosphate potential) when UCP is inactive and H^+ leak is negligible. In the presence of free fatty acids (FFA) that activate UCP, $\Delta\mu H^+$ is shared between UCP and ATP synthase and, if the latter is inhibited by oligomycin, $\Delta\mu H^+$ is dissipated mainly through UCP activity as FFA are present.

RESPIRATORY ACTIVITIES VERSUS CONTRIBUTIONS

Respiratory activities measured with isolated mitochondria can be sustained by ATP synthesis, UCP activity and AOx activity. Measurements made in situations in which one of the activities is functioning while the other two are blocked do not reflect the true contributions of each pathway to the overall state 3 respiration as any change in one inevitably affects the others. Therefore, we have devised a method based on the non-phosphorylating property of the free energy-dissipating pathways (AOx and UCP) to determine contributions of each pathway into overall state 3 respiration (Sluse *et al.*, 1998a; Sluse and Jarmuszkiewicz, 2000). However, even if respiratory rate measurements in the presence of specific inhibitors do not reflect true contributions of each pathway they do allow relative comparisons.

AOx-UCP CONNECTION IN TOMATO FRUIT (*Lycopersicon esculentum*)

CN-resistant respiration represents AOx activity and the linoleic acid (LA)-induced (BHAM + oligomycin)-resistant respiration represents part of tomato *Lycopersicon esculentum* UCP (LeUCP) activity. These activities were measured for various linoleic acid (LA) concentrations with tomato mitochondria fully depleted of

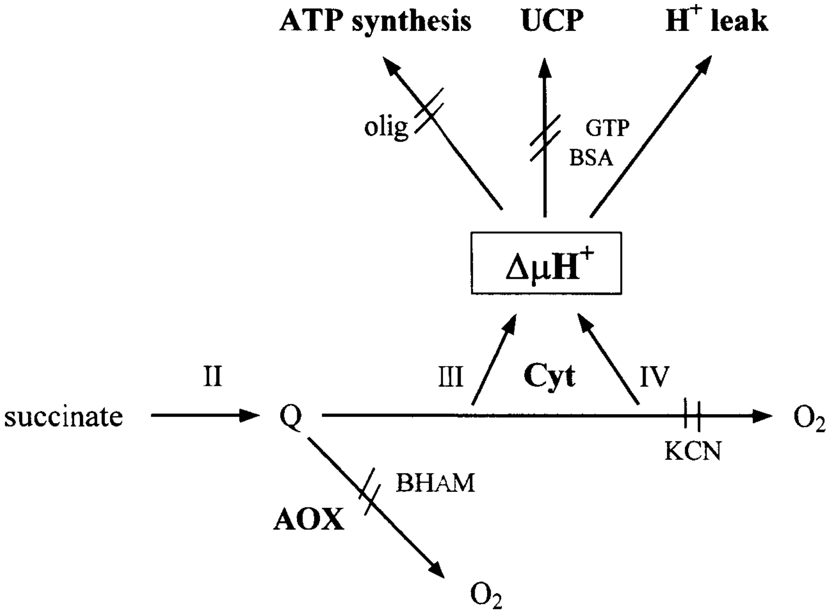

Fig. 1. Respiratory network in tomato mitochondria. Complex II, succinate dehydrogenase; complex III, cytochrome *bc*$_1$; complex IV, cytochrome *c* oxidase; AOx, alternative oxidase; Cyt, cytochrome pathway; Q, ubiquinone; UCP, plant uncoupling protein; $\Delta\mu H^+$, proton electrochemical gradient built by the cytochrome pathway; BHAM, benzohydroxamic acid; KCN, potassium cyanide; olig., oligomycin; GTP, guanosine triphosphate; BSA, bovine serum albumin.

free fatty acids (FFA) as described in the legend of Fig. 2. The LA-induced respiration increases with increasing concentration of LA and 50% maximal stimulation is reached at 10 μM. The CN-resistant respiration in the presence of dithiothreitol (DTT) and pyruvate, activators of AOx, decreases with increasing concentration of LA (Sluse *et al.*, 1998b). Fifty percent inhibition is reached at LA concentration around 4 μM. This experiment describes how an increase in FFA level can affect both of the energy-dissipating systems but in opposite directions. These results are important because they show how AOx can be progressively switched off by an increase in the FFA content in cells and they also indicate that AOx and LeUCP never work together at their maximal activity. It can be proposed that the two energy-dissipating enzymes work sequentially during cell life of tomato fruits.

EVOLUTION OF AOx AND LeUCP ACTIVITIES DURING RIPENING

As AOx and LeUCP activities seem to exclude mutually *in vitro*, they could work sequentially during the life of plant cells according to their particular physiological state. Ripening of fruits provides an interesting model to study a relationship between AOx and UCP as thermogenesis occurs during ripening (Kumar *et al.*, 1990; Kumar and Sinha, 1992) and FFA concentration increases in the post-growth stage (Güçlü *et al.*, 1989; Rouet-Mayer *et al.*, 1995).

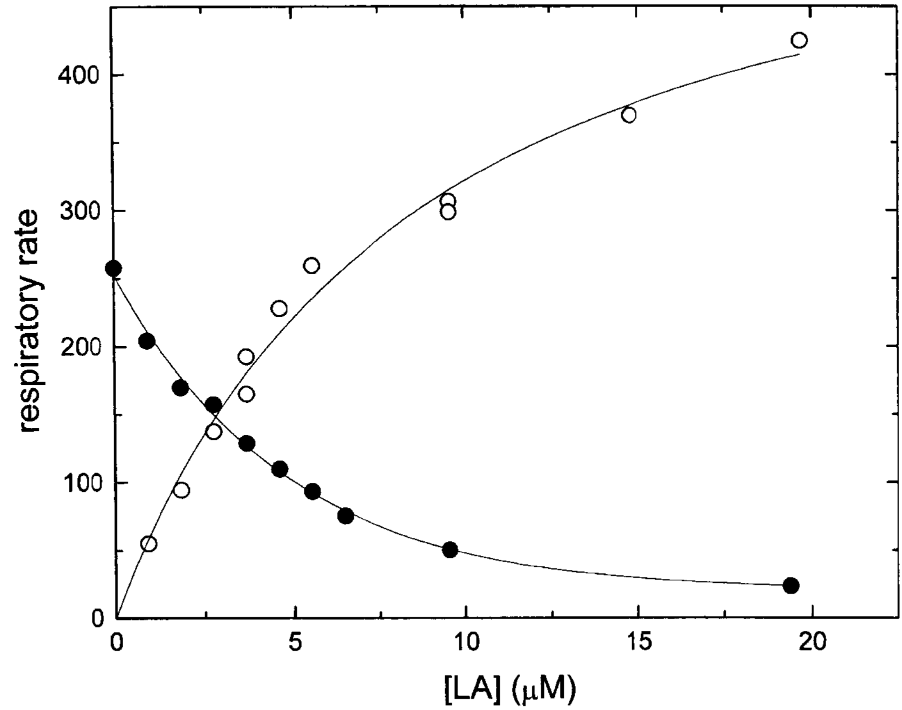

Fig. 2. CN-resistant respiration and LA-induced respiration versus LA concentration. Fully depleted of FFA green tomato mitochondria (Sluse *et al.*, 1998a) were incubated with 10 mM succinate, 5 μM rotenone, 2.5 μg of oligomycin/mg protein, and 0.17 mM ATP. Cyanide-resistant respiration (●) (+1 mM KCN) was measured in the presence of 1 mM dithiothreitol (DTT) plus 0.15 mM pyruvate. LA-induced respiration (○) was measured in the presence of 2 mM BHAM. Increasing concentrations of LA (1.2–20 μM) were obtained by successive additions when the steady-state respiration rate was achieved.

The evolution of ATP synthesis-sustained respiration, AOx-sustained respiration, and LeUCP-sustained respiration can be measured in mitochondria isolated from tomato fruit during post-harvest ripening. ATP synthesis-sustained respiration was measured in state 3 in the presence of succinate (+rotenone) as substrate, BHAM and GTP + BSA as inhibitors of AOx and LeUCP, respectively. When plotted against the stage of ripeness, it decreases up to the orange stage then stabilizes between the orange and red stages (Fig. 3A, ATP synthase). The AOx-(cyanide-resistant)-sustained respiration was measured in state 3 in the presence of cyanide and GTP + BSA as inhibitors of the cytochrome pathway and LeUCP, respectively. When plotted against the stage of ripeness, it decreases from the green to the orange stage and then stabilizes (Fig. 3A, AOx). LeUCP-sustained respiration was measured in state 4 in the presence of oligomycin and BHAM, as inhibitors of ATP synthase and AOx, respectively, and of 10 μM LA. The respiratory rate after LA addition represents LeUCP-sustained respiration plus H^+ leak (negligible in the presence of LA due to LA-induced drop in state 4 membrane potential). With the stage of

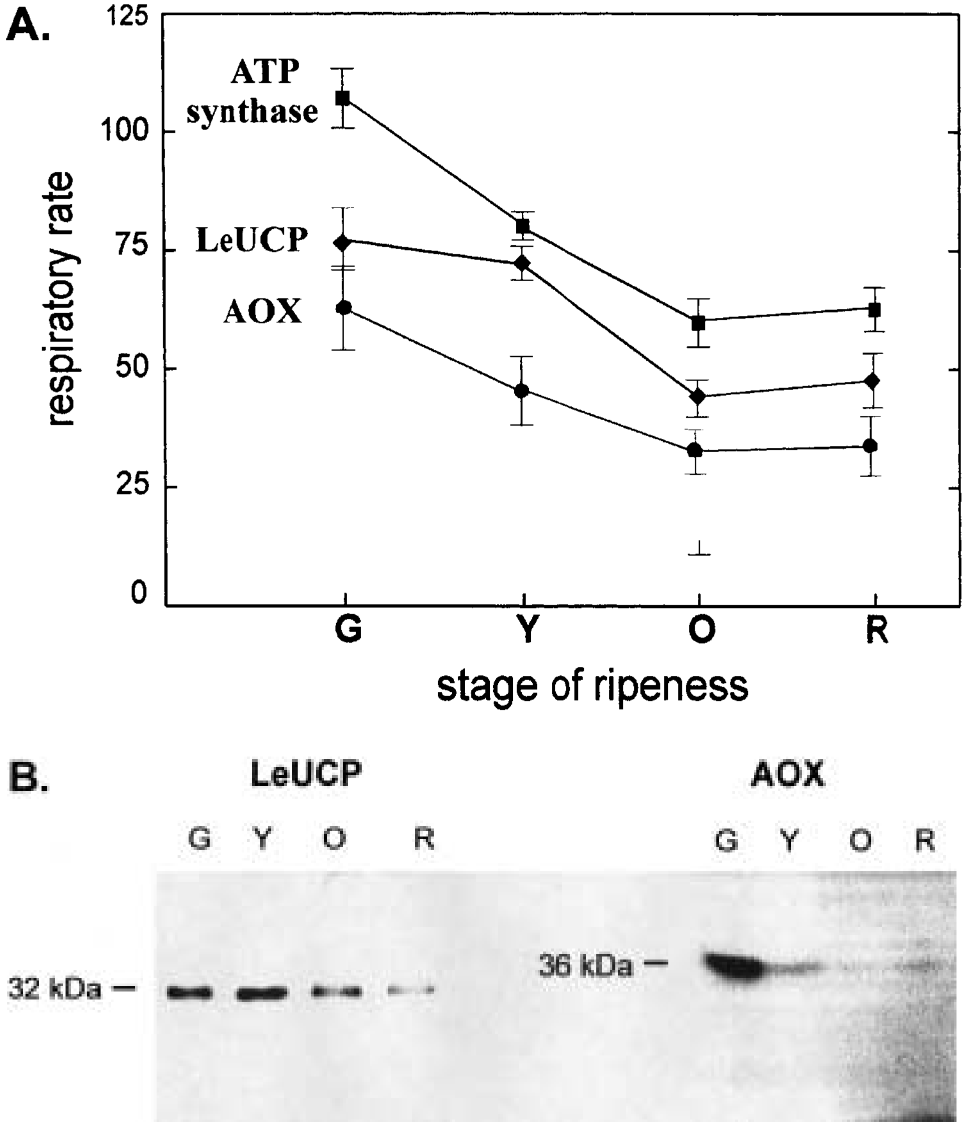

Fig. 3. Analysis of respiratory activities and immunodetection of AOx and LeUCP during tomato fruit ripening. A. Fully depleted of FFA green tomato mitochondria were incubated with 10 mM succinate, 5 μM rotenone, and 0.17 mM ATP (Almeida *et al.*, 1999). Respiratory rate sustained by ATP synthase activity (■) was measured in the presence of 0.5% BSA, 1 mM GTP, and 2 mM ADP (for state 3). Respiratory rate sustained by LA-induced LeUCP activity (◆) was measured in state 4 in the presence of 2.5 μg of oligomycin/mg protein, 2 mM BHAM, and 10 μM LA. Respiratory rate sustained by cyanide-resistant AOx activity (●) was measured in the presence of 1.5 mM KCN, 0.15 mM pyruvate, 1 mM DTT, 0.5% BSA, 1 mM GTP, and 2.5 μg of oligomycin/mg protein ATP synthase-, LeUCP- and AOx-sustained respiration was examined at different stages of ripeness (G, green; yellow, Y; orange, O; red, R). B. Immunoblot analysis of tomato mitochondrial proteins at different stages of ripeness. Monoclonal antibodies against the *S. guttatum* AOx and polyclonal antibodies against the *S. tuberosum* UCP were used as described by Almeida *et al.* (1999).

ripeness, LeUCP-sustained respiration only significantly decreases between the yellow and orange stages and then stabilizes (Fig. 3A, LeUCP).

Immunoblotting of mitochondrial proteins of tomato fruit allowed detection of AOx and LeUCP proteins. Figure 3B clearly shows that both proteins are simultaneously present in green mature tomato fruit mitochondria. The level of immunodetectable AOx decreases with ripening from green stage forward, and parallels the decrease in AOx-sustained respiration. Changes in LeUCP protein levels are less pronounced, and a decrease occurs between the yellow and orange stages, as it is the case for LeUCP sustained respiration. These results clearly indicate (1) a regulation of AOx activity through a decrease in protein expression during tomato postharvest ripening, (2) a significant decrease in LeUCP protein expression only at the orange stage that parallels LeUCP activity, and (3) a sequential activity of AOx and LeUCP during tomato ripening. Therefore, AOx would be active mainly during the growing period whereas LeUCP would start working in post-growth stage.

POTENTIAL ROLE OF AOx AND UCP: ENERGY METABOLISM BALANCE

As mentioned above, the obvious physiological role of energy-dissipating pathways is for heat generation in thermogenic tissues. Energy-dissipating systems could have the more subtle role in energy metabolism control working as safety valves when overloads in redox potential or/and in phosphate potential occur. AOx could work as valve in situations that lead to an increase in reducing power and in phosphate potential in the cell. Indeed, if reducing power increases, electron partitioning to AOx will increase due to AOx activation by (1) increase in ubiquinol (substrate), (2) increase in NADPH (more active AOx dimer), (3) increase in pyruvate (activator of AOx). If phosphate potential is high (high ATP/ADP ratio), the decrease of electron flux into the cytochrome pathway due to proton electrochemical potential back pressure will increase the electron partitioning to AOx as an indirect consequence of respiratory control. On the contrary, UCP is able to consume the H^+ gradient directly and consequently decrease phosphate potential when activated by FFA as well as redox potential. Activation of UCP could lead to full uncoupling when FFA are present at a sufficient concentration but it could also manage partial uncoupling and correct overload of potential when FFA concentration is low.

Excess redox potential and excess phosphate potential are consequences of imbalance between reducing substrate supply and energy and carbon demand for biosynthesis both being coupled by the respiratory chain activity (Fig. 4). As operation of AOx can directly decrease the reducing power rise and operation of UCP can directly induce drop in the phosphate potential, they could theoretically correct the imbalance. However, according to our observations simultaneous presence of both proteins, AOx and UCP, in mitochondria at a given moment of cell life does not mean either simultaneous activity or simultaneous permanent presence. According to results obtained for tomato fruit ripening, AOx would be active mainly during high biosynthetic activities in the cell, i.e., during plant growth and development, providing a safety balance between redox potential, phosphate potential, and biosynthesis demand, while UCP, even if present, would be functionally silent or at

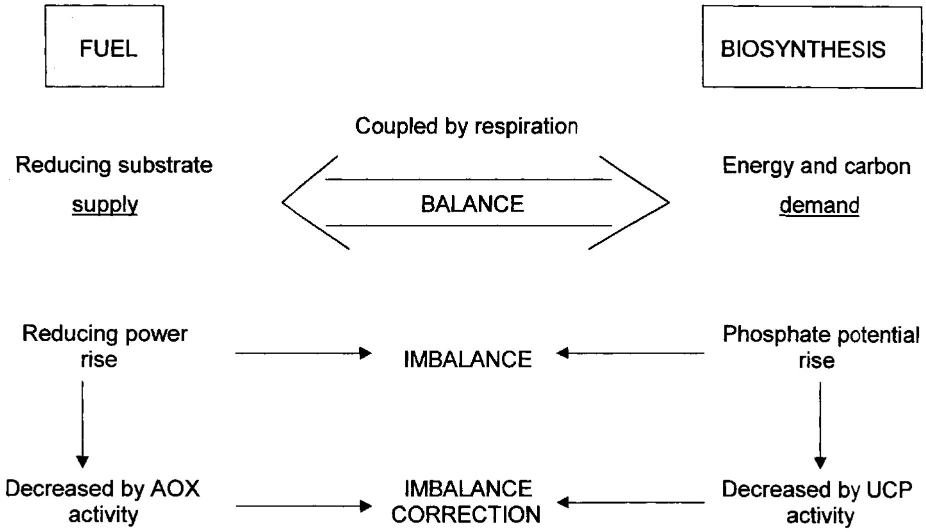

Fig. 4. Energy balance of the cell. Proposed role of AOx and UCP in energy imbalance correction of cell metabolism.

the very most weakly active providing mitochondria with mild uncoupling as a safety valve. With the FFA concentration increase in post-growth stages like fruit ripening, but perhaps also like senescence and flowering, UCP would start working at full rate providing a mechanism for termination of biosynthetic processes through the dissipation of oxidative phosphorylation efficiency in parallel with heat production as an unavoidable event.

NON-THERMOGENIC TISSUE HEAT PRODUCTION: THERMAL BALANCE AND ITS LIMIT

Heat production as a side event of free-energy dissipation in "non-thermogenic tissues" does not mean a useless event. Indeed, if linked to increase in temperature, heat production has overall effect on enzymes' activities particularly in poikilotherms. Therefore, a "thermal balance" could occur between energy-conserving processes on one side and energy-dissipating processes on the other side, the first providing elements for biosynthetic activities and the latter providing better intercellular thermal surroundings. This proposal relates to observations concerning cold stress responses. It is well known that cold stress induces an increase in AOx activity and protein expression in plants (Purvis, 1985; Elthon *et al.*, 1986; Stewart *et al.*, 1990; Vanlerberghe and McIntosh, 1992; Duque and Arrabaça, 1999). Recently, it has been shown that cold exposure induces UCP protein expression in potato mitochondria (Laloi *et al.*, 1997; Nantes *et al.*, 1999). It also relates to some adaptations of antarctic fishes. The icefish (*Channichthys rhinoceratus*), a predator which lives at around 0°C, possesses surprising heart mitochondria with an uncoupled succinate-oxidizing pathway that produces heat from succinate accumulated during anaerobiosis (Feller *et al.*, 1989). However, the concept of "thermal balance" as a response to environmental-thermal pressure cannot be extended to unicellulars because their microscopic size that does not allow increase in temperature. Indeed, their surface/volume ratio is so high that it excludes the possibility of any steady-state local heating because heat diffusion is too fast due to thermal conductivity of medium. This is also true for mitochondria (Breidenbach *et al.*, 1997). Moreover, the actual link between free-energy dissipation with heat production and with increase in temperature of tissue or organism (thermogenesis) is not only restricted by a lower-limit size but also by the true effect of the free-energy dissipating systems activation on the heat production rate in a given constant steady-state metabolism activity *in vivo*. Indeed, this last point implies that different oxidative pathways have large differences in enthalpy changes, more exothermic for AOx- and UCP-sustained respiration. However, a complete shift of electron flux from the cytochrome pathway to AOx would increase heat production by only 6% for the same oxygen uptake (Breidenbach *et al.*, 1997) and this difference would be almost negligible in intermediary situations. Thus, combined calorimetric and respirometric data invalidate the large difference in enthalpy change supposed above. Therefore, the increase in heat production rate observed after low-temperature exposure of chilling-sensitive plants (Moynihan *et al.*, 1995) must come from an overall increase in the rate of oxidative reactions mainly due to an increase in AOx and UCP protein amount (translational up-regulation) (Almeida *et al.*, 1999) or a strong posttranslational activation (for AOx). Thus, increase in heat production rate and in temperature, observed during reproduction-related thermogenesis in aroids, is mainly due to a tremendous increase in oxidation rate of substrates during reproductive processes. Of course, for an isolated mitochondrial suspension in state 3 in the absence of ATP consuming processes, heat production rate, at the same oxygen uptake, will increase if respiration shifts from cytochrome pathway to AOx because no overall steady-state exists and enthalpy of ATP hydrolysis is not included in the balance. But nevertheless, no "local mitochondrial" increase in temperature could be detected. Thermogenesis through UCP is submitted to the same restrictions linked to thermal conductivity (size of organism) and to net increase in overall steady-state oxygen uptake (translational and posttranslational up-regulation). For example, in the case of green mature tomato mitochondria, maximal state 3 respiration (in the presence of benzohydrohamate, BHAM, an inhibitor of AOx), uncoupled respiration (with FCCP as uncoupler, +BHAM) and maximally LA-stimulated respiration (in the presence of BHAM) are the same (Jarmuszkiewicz *et al.*, 1998). This means that the overall oxygen uptake is kinetically limited by the respiratory chain that, *in vivo* at a given moment, will also limit a thermogenesis increase by shifting from fully phosphorylating respiration to respiration fully uncoupled by LA.

CONCLUSIONS

The two energy-dissipating systems present in plant and *Acanthamoeba* mitochondria lead to the same final effect that is a decrease in ATP synthesis accompanied by heat production. The latter is used for clear physiological purposes in specialized thermogenic tissue and may be only a side event in other tissues. In non-thermogenic tissues of plants, AOx and UCP activities may have a regulatory role in the balance of energy metabolism, AOx being mainly devoted to growth and development phenomena and UCP to post-growth stages when FFA concentration increases. These considerations are based on the functional connection between plant AOx and UCP through FFA and on their sequential work during ripening of tomato fruits. The concept of "energy balance" may be associated with the more daring concept of "thermal balance" mainly in poikilotherms which have to adapt to thermal conditions of their surroundings. In the latter concept, UCP and AOx could be considered as response proteins to cold exposure (translational regulation) allowing increase in oxygen uptake and some improvement of biosynthesis and growth at low external temperature. The cost of that improvement would be paid by the so-called free-energy "dissipation."

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